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AWARD NUMBER: W81XWH-08-1-0404

TITLE: Met (HGF Receptor) in Breast Cancer

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REPORT DATE: September 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 September 2009	Annual Summary	1 Sep 2008 – 31 Aug 2009

4. TITLE AND SUBTITLE

Met (HGF Receptor) in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0404

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University
New Haven, CT 06510

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The hepatocyte growth factor (HGF)/Met signaling pathway has been shown to be important for stimulating cell proliferation, motility, invasion and metastasis. Recent work from our lab has identified a 60 kDa fragment from the carboxy-terminal domain of Met that localizes to the nucleus. Preliminary data from our also indicates that Met is translocated to the nucleus during in vitro wound healing of epithelial sheets of cells. Because several pharmaceutical companies are currently developing Met-based therapies, it becomes even more important to gain an understanding of the role of nuclear Met, especially whether or not it may be contributing to invasion and metastasis. To date, no studies have been conducted to understand this aspect of Met function. Therefore the objectives of my proposal are to assess the role of Met in a model of epithelial-mesenchymal transition (EMT), identify key residues in the Met receptor necessary for nuclear translocation, and determine the functional role of Met in the nucleus.

15. SUBJECT TERMS
Met, HGF receptor, wound healing

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	18	19b. TELEPHONE NUMBER *(include area code)*

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Introduction:
The hepatocyte growth factor (HGF)/Met signaling pathway has been shown to be important for stimulating cell proliferation, motility, invasion and metastasis. Activating Met mutations are thought to contribute to the cause of several cancer types including papillary renal carcinoma, lung cancers, head and neck cancers and gastric cancer (1). Increases in Met expression have also been correlated with increased aggressiveness of cancer (2) and Met expression is both highest and strongly nuclear at the invasive front in breast carcinoma (3). Recent work from our lab has also demonstrated patients with high levels of nuclear Met have a worse prognosis even in lymph node negative breast cancer (4, 5) and has also identified a 60 kDa fragment from the carboxy-terminal domain of Met that localizes to the nucleus. Preliminary data from our also indicates that Met is translocated to the nucleus during *in vitro* wound healing of epithelial sheets of cells. Because of this, the underlying hypothesis of this proposal is: the translocation of Met to the nucleus leads to interactions and functions contributing to cancer progression by promoting epithelial-mesenchymal transition. Because several pharmaceutical companies are currently developing Met-based therapies, it becomes even more important to gain an understanding of the role of nuclear Met, especially whether or not it may be contributing to invasion and metastasis. To date, no studies have been conducted to understand this aspect of Met function.

Body:
The goal of this research is to assess the role of the Met receptor in a model of epithelial-mesenchymal transition (EMT) in addition to identifying key residues of the Met receptor necessary for nuclear translocation and to determine the functional role of Met in the nucleus.

The original statement of work was the following:

Task 1: Identification of genes regulated by nuclear Met
a. Perform ChIP on MCF7 breast cancer and HMEC normal breast cell lines under normal epithelial conditions and after triggering of a mesenchymal transition
b. Send isolated DNA fragments to the 454 company for sequencing
c. Align sequencing results and determine frequency distributions for promoter regions

Timeline: Months 1-6

Task 2: Validation of ChIP-454 promoter sequences
a. Clone promoter sequences into luciferase reporter plasmids
b. Transfect into cell lines with strong nuclear Met and measure luciferase expression
c. Transfect into cell lines with membranous Met inhibited with PHA and measure luciferase expression

Timeline: Months 5-12

Task 3: Determine the role of Met in epithelial-mesenchymal transitions
a. Use siRNA to knock down Met expression in LIM1863 cells and a scrambled RNA as a control for specificity and/or treat the cells with PHA to inhibit Met kinase activity and nuclear translocation
b. Confirm specific inhibition of Met expression and/or activity by western blot
c. Treat LIM1863 organiods with recombinant TGFb and TNFa in the presence of Met inhibition compared to control treated with scrambled RNA or not inhibited with PHA

d. Verify EMT in control cells by loss of E-cadherin and migration of cells from the organoid to form a monolayer and use these markers to determine if Met inhibition prevents this transition

Timeline: Months 11-24

Task 4: Generate stable MCF7 and CaCo2 cell lines expressing tagged Met

a. Clone full-length Met-GFP and Met-HA epitope tag into retroviral vectors
b. Transfect into HEK293T cells to obtain packaged viral particles and use to generate MCF7 and CaCo2 stable cell lines expressing these constructs
c. Perform a wound healing assay with each of the stable lines and determine subcellular localization of full-length Met-GFP or Met-HA

Timeline: Months 23-30

Task 5: Identify residues necessary for nuclear translocation of Met

a. Use site-directed mutagenesis on the full-length Met construct to create a deletion series of the juxtamembrane domain and full-length constructs with alanine substitutions at residues Y1349, Y1356, or both Y1349 and Y1356
b. Transfect into HEK293T cells to obtain packaged viral particles and use to generate MCF7 and CaCo2 stable cell line clones expressing each of the above constructs
c. Perform a wound healing assay with each of the stable lines and determine the subcellular localization of each mutant construct

Timeline: Months 26-36

The goal of Task 1 was to identify genes regulated by nuclear Met through 454-sequencing of DNA isolated by ChIP of wounded versus un-wounded cells. This was attempted at the start of this project, however the 454 company was unable to amplify from the isolated DNA we sent. While unfortunate, we came to the realization at this time that the manner we were using to generate nuclear Met (wounding of the cellular monolayer) would not generate uniform transcriptional changes in all of the cells as only cells at the wound edge expressed nuclear Met (Figure 3). In addition, a number of the transcriptional changes we may have identified would have been as a result of the wound healing process and not necessarily directly related to the presence of nuclear Met, decreasing the chance they would be verified as Met targets in Task 2. To increase the likelihood that we would successfully identify genes modulated by nuclear Met, we have focused our initial efforts on Task 4: generation of stable cell lines expressing Met tagged with GFP. Having cell lines stably expressing either full-length Met-GFP, the cytoplasmic domain of Met known to be necessary for nuclear localization, and a truncated c-terminal domain of Met previously shown to by our lab to be excluded from the nucleus (6) would allow us to compare transcriptional profiles of MCF7 lines either expressing nuclear Met or Met excluded from the nucleus. Many months of this past year have been focused on generating these cell lines. First attempts to generate stable lines were made using the constructs previously generated in the lab (6) for transient transfections. MCF7 cells were transfected with Lipofectimine and grown in selection media with G418 theoretically selecting for cells maintaining expression of Met-GFP. Generation of MCF7 cells stably expressing the GFP control was successful, but all attempts at obtaining stable expression of Met-GFP using this method were not successful. We then moved to clone Met-GFP into viral vectors to instead generate stable expression by taking advantage of the lentiviral/retroviral infection method. We

obtained a pHR'-GFP expression vector from Addgene with GFP under the control of a CMV promoter. We used two rounds of mutagenesis to generate a unique restriction site between the CMV and GFP that allowed us to insert the coding sequence for Met. We successfully generated a clone containing the in-frame sequence of Met-GFP under the CMV promoter and are currently packaging virus in HEK293 cells to infect MCF7 cells with this construct. Because the pHR' vector does not contain an additional selection marker other than GFP expression, we are also working to clone the Met-GFP sequence into a pBABE vector containing the additional puromyocin selection.

Task 3 was to determine the role of Met in epithelial-mesenchymal transitions. Before moving to a 3-dimensional model system we have focused first on analyzing the role of Met in wound healing of cellular monolayers. The first step in this process was to develop a device that allowed for reproducible wounding of the cells to standardize the conditions where we observed nuclear Met. Our method for wounding is a stabilized 20 μl pipet tip (Figure 1A). Phase contrast images are taken at the initial and final time points (Figure 1B), are thresh-holded in Photoshop (Figure 1C), and the wound area is measured using a Java script (Figure 1D & E). We have also demonstrated that as expected, HGF treatment (50 ng/ml) and therefore Met activation increases the healing response or Met inhibition with the Pfizer compound PHA-66552 prevents wound healing. Phase contrast examples of this are shown in Figure 2 for H1650 cells and quantified in Figure 3. Similarly, BT20, A431, and CaCo2 cell lines also demonstrate the same patterns when quantified and results for all cell lines are shown in Figure 3. In addition to the overall wound healing response dependency on Met expression, immunofluorescence staining of H1650 cells shows that Met is localized to the nucleus at the wound edge. 60x deconvolution images are shown in Figure 4 for H1650 cells treated with HGF and stained 8 hours after wounding with CVD13, a c-terminal Met Ab. Interestingly, immunofluorescence staining of H1650 cells 8 hours after wounding also shows that nuclear Met is also inhibited by PHA treatement, even in the presence of HGF (Figure 5). We have also observed nuclear Met at the wound edge in a number of other cell lines and an example in A431 cells is shown in Figure 6. Our next question was whether or not nuclear Met was activated. To address this we also stained H1650 cells wounded for 8 hours with a phospho-Met antibody specific to residues 1234/1235 of the tyrosine kinase domain and found nuclear localization again at the wound edge either in serum conditions or with addition of exogenous HGF (Figure 7).

In addition, we have also generated shRNA knockdown of Met through lentiviral infection of CaCo2, A431, and H1650 cell lines. Knockdown of Met eliminated the increase in healing we observed when the cells were treated with HGF and is shown for the H1650 cell line in Figure 8a. Met knockdown was confirmed by Western blot and is shown in Figure 8b. Because we see nuclear Met expression in untreated (serum only, no exogenous HGF) cells (Figures 5-7) we are in the process of determining if nuclear Met requires activation of the receptor by HGF. To address this we first analyzed the level of HGF mRNA expression in a panel of cell lines (Figure 9a) and determined that H1650 cells do express HGF mRNA and also have basal levels of activated Met as shown by Western blot in Figure 10a for phospho-Met in H1650 cells. It is important to note that this activation is not likely to be due to endogenous HGF expression alone as this cell line has an activating mutation in EGFR that is also likely playing a role in Met activation. Therefore we searched in the literature for a cell line reported to have low endogenous mRNA expression of HGF and found A549 cells were reported to have this

characteristic. We first wanted to validate that this cell line does not express HGF, however as shown with two independent primer pairs in Figures 9B and 9c, A549 cells do still have low levels of HGF mRNA. Even though these cells do still express low levels of HGF mRNA, we determined the level of endogenous Met activation by Western blot and show in Figure 10b that there are very low phosho-Met levels in serum growth conditions, but that exogenous HGF treatment (50 ng/mL) is able to activate the Met receptor.

No work has been completed on Task 5 to date. Completion of viral infection of MCF7 cells with Met-GFP to generate stable cell lines will allow us to determine if this method will be feasible for assessing the mutation constructs.

Key Research Accomplishments:

- Generation of pHR'-Met-GFP
- Reproducible method for wound healing and quantification
- Nuclear Met is observed at the leading edge of the wounds
- Nuclear Met is inhibited in H1650 cells when treated with a small molecule inhibitor
- Nuclear Met is phosphorylated in H1650 cells

Reportable Outcomes:

Presentation: Yale University Department of Pathology Research in Progress

Conclusion:

In conclusion, my results to date demonstrate nuclear Met is present in the wound edge and in addition is phosphorylated. This lead to the question if Met must first be activated by HGF to translocate to the nucleus. We have identified a cell line, A549 that has very low levels of activated Met when grown in serum conditions and are presently determining the localization patterns of Met by immunofluorescence. If we observe nuclear Met in the absence of exogenous HGF, then we can conclude that HGF addition is not required for nuclear localization. The nuclear localization of Met at the wound edge has also raised a number of questions we are currently pursuing including if the nuclear localization is a function of migration, proliferation, or loss of cell-cell contacts and E-cadherin. The generation of MCF7 cells stably expressing Met-GFP will allow us to track the localization of Met in live cells after wounding and generation of cell lines expressing the predominately nuclear or cytoplasmic Met constructs will allow us to determine what the functional role of nuclear Met is. Determining the function of nuclear Met is extremely important as a number of pharmaceutical companies are currently in the process of developing cancer therapies based on inhibition of Met. Additional work from our lab has published that, in a cohort of 640 cases of invasive breast cancer on a tissue microarray, high levels of nuclear Met were associated with poor survival (4). This highlights the functional relevance of nuclear Met and understanding the mechanism and function behind nuclear Met could provide additional insight as to how this receptor functions in cancer progression.

References:

1. Zou H, Li Q, Lee J, et al. An Orally Available Small-Molecule Inhibitor of c-Met, PF-2341066, Exhibits Cytoreductive Antitumor Efficacy through Antiproliferative and Antiangiogenic Mechanisms. Cancer Research 2007; 67: 4408-17.

2. Jin L, Fuchs A, Schnitt SJ, et al. Expression of scatter factor and c-met receptor in benign and malignant breast tissue. Cancer 1997; 79: 749-60.
3. Edakuni G, Sasatomi E, Satoh T, Tokunaga O, Miyazaki K. Expression of the hepatocyte growth factor/c-Met pathway is increased at the cancer front in breast carcinoma. Pathol Int 2001; 51: 172-8.
4. Pozner-Moulis S, Cregger M, Camp RL, Rimm DL. Antibody validation by quantitative analysis of protein expression using expression of Met in breast cancer as a model. Lab Invest 2007; 87: 251-60.
5. Camp RL, Rimm EB, Rimm DL. Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma. Cancer 1999; 86: 2259-65.
6. Pozner-Moulis S, Pappas DJ, Rimm DL. Met, the hepatocyte growth factor receptor, localizes to the nucleus in cells at low density. Cancer Research 2006; 66: 7976-82.

Appendices & Supporting Data:

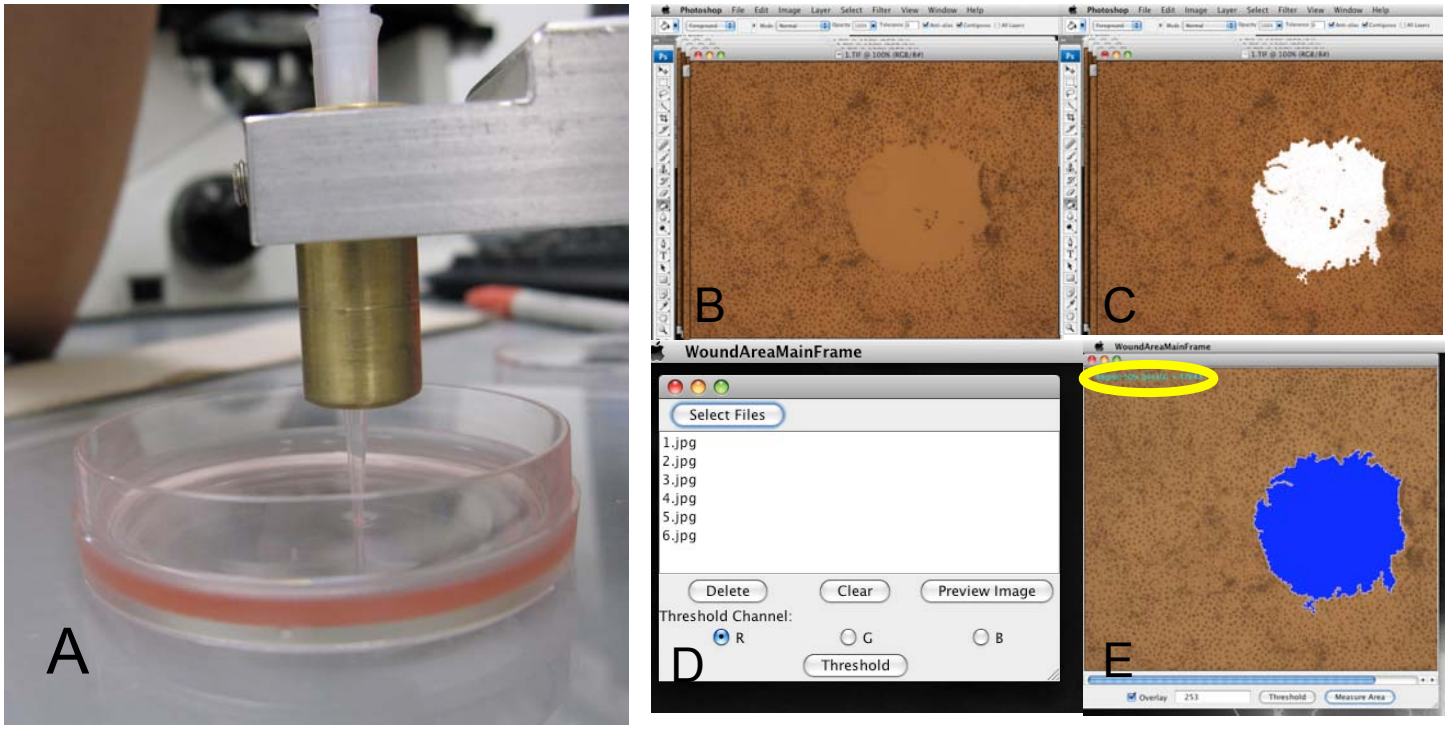

Figure 1: Wound healing method. A: 20 µl pipet tip is used to create circular wounds on a coverslip of confluent cells. B: Phase contrast images are taken of wounds at time 0 and final time point for each experiment. C: Wound area is filled with white using photoshop to allow for easy thresh-holding. D: WoundArea java program is used to select experiment files containing wound images. E: Image is thresh-holded to select the wound area and the total area (pixels) is displayed in the top left of the window (circled in yellow).

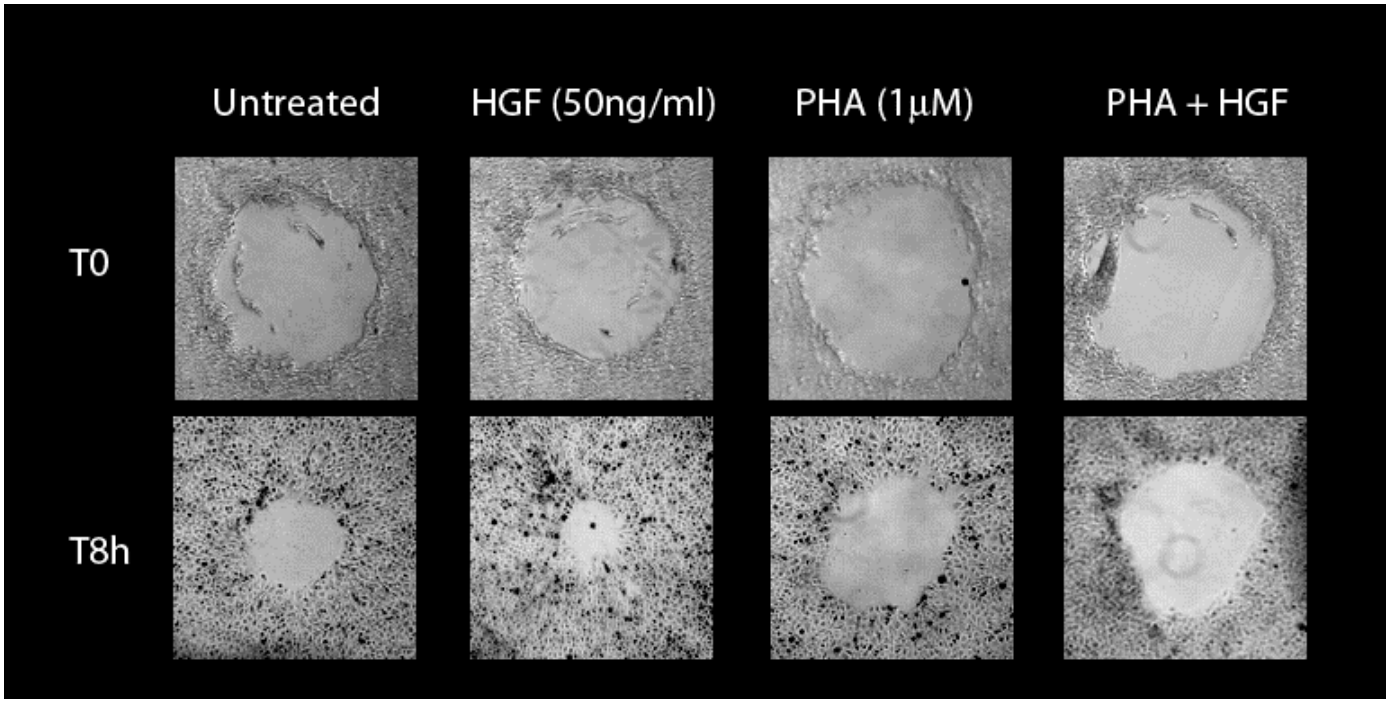

Figure 2. Wound healing of H1650 cells is increased upon HGF treatment and inhibited upon pretreatment of cells with PHA for 2 hours prior to wounding. Phase contrast images are shown of Time 0 (Top row) and Time 8 hours (Bottom row) for each condition: Untreated, HGF (50 ng/ml), PHA (1μM), and PHA (1μM) with HGF (50ng/ml) addition at time of wounding.

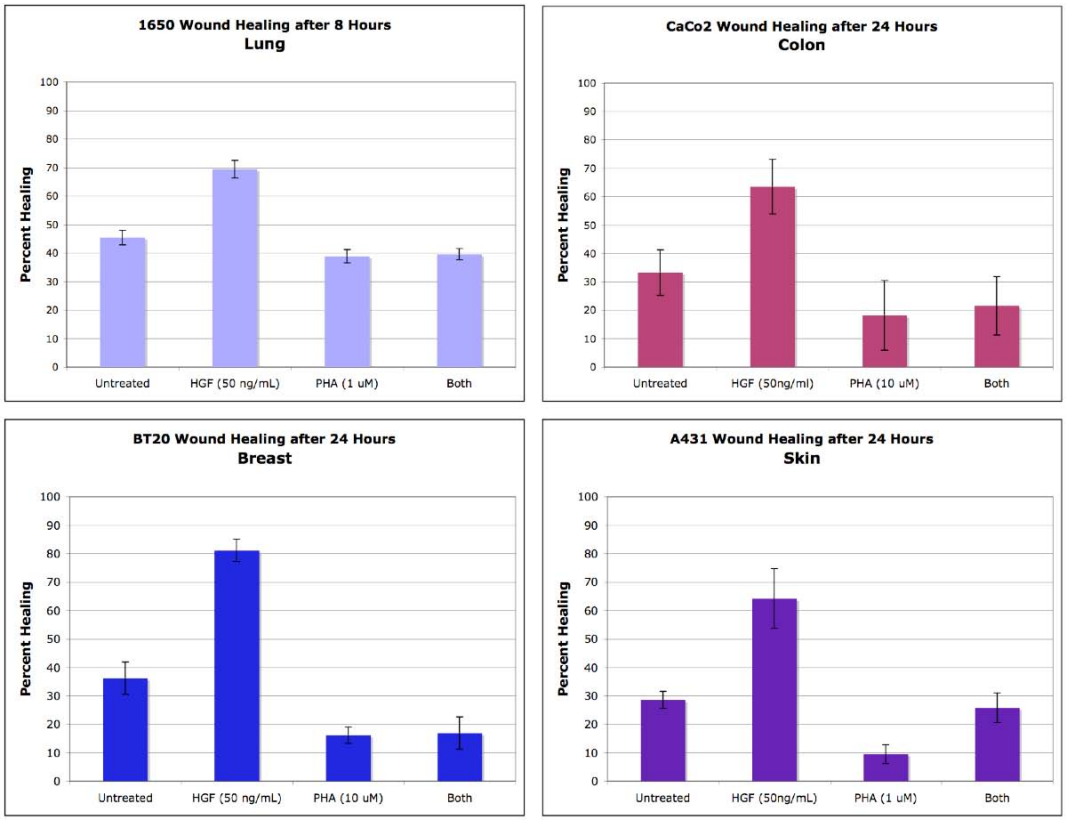

Figure 3. Total wound healing response in H1650, CaCo2, BT20, and A431 cell lines. Error bars represent standard error of the mean.

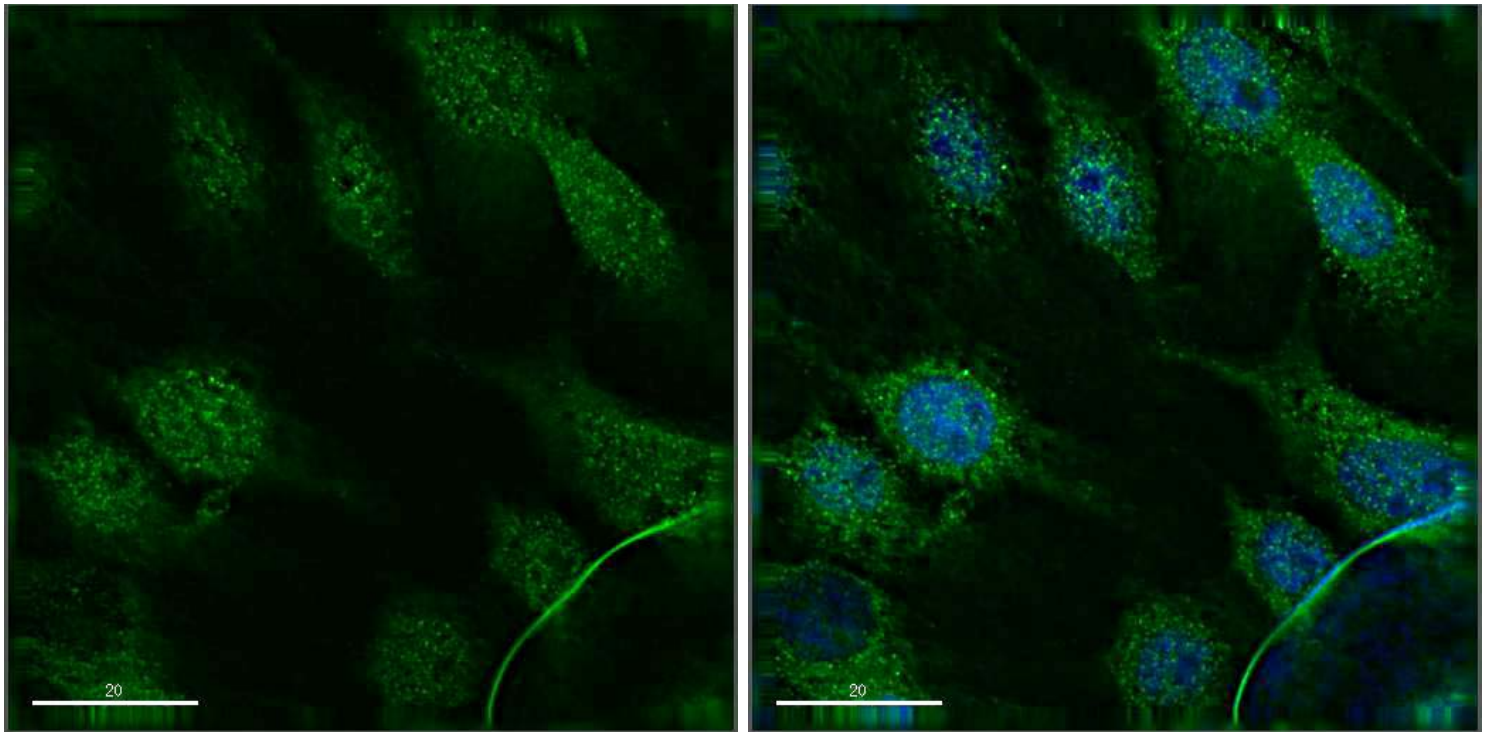

Figure 4. H1650 cells have nuclear Met at the wound edge after HGF treatment 8 hours post wounding. Green – stained for Met (CVD13 1:500) Blue – represents DAPI staining. Images are taken at 60x using a deconvolution microscope. Left: Met staining alone. Right: Met staining with DAPI overlay.

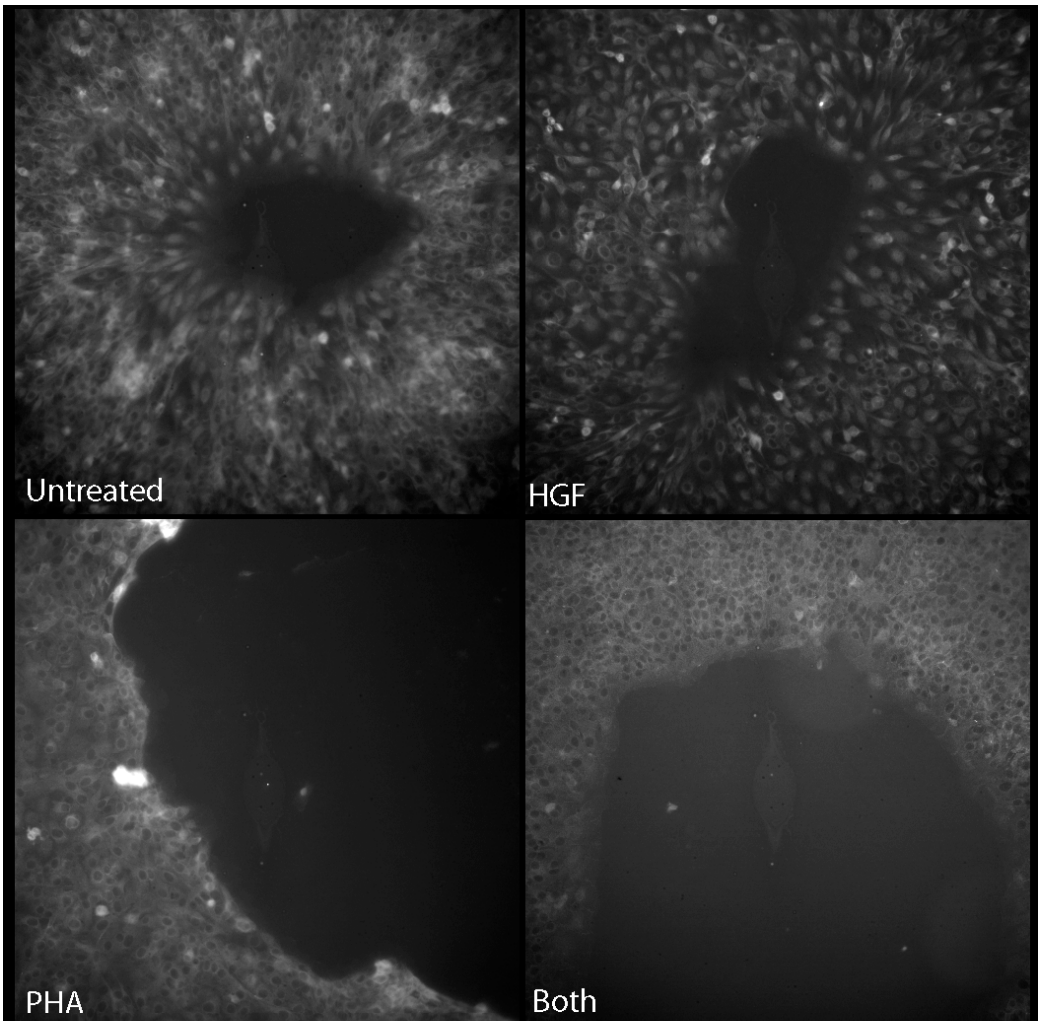

Figure 5. Nuclear Met is present in the invasive front of wounded H1650 cells untreated and treated with HGF (50ng/ml) for 8 hours. Top left: Untreated cells. Top right, HGF stimulation. Bottom left, PHA inhibition 2 hours prior to wounding (1mM). Bottom right, PHA treatment 2 hours prior to addition of HGF and wounding. Cells were stained with Met Ab CVD13 (Invitrogen) 1:500.

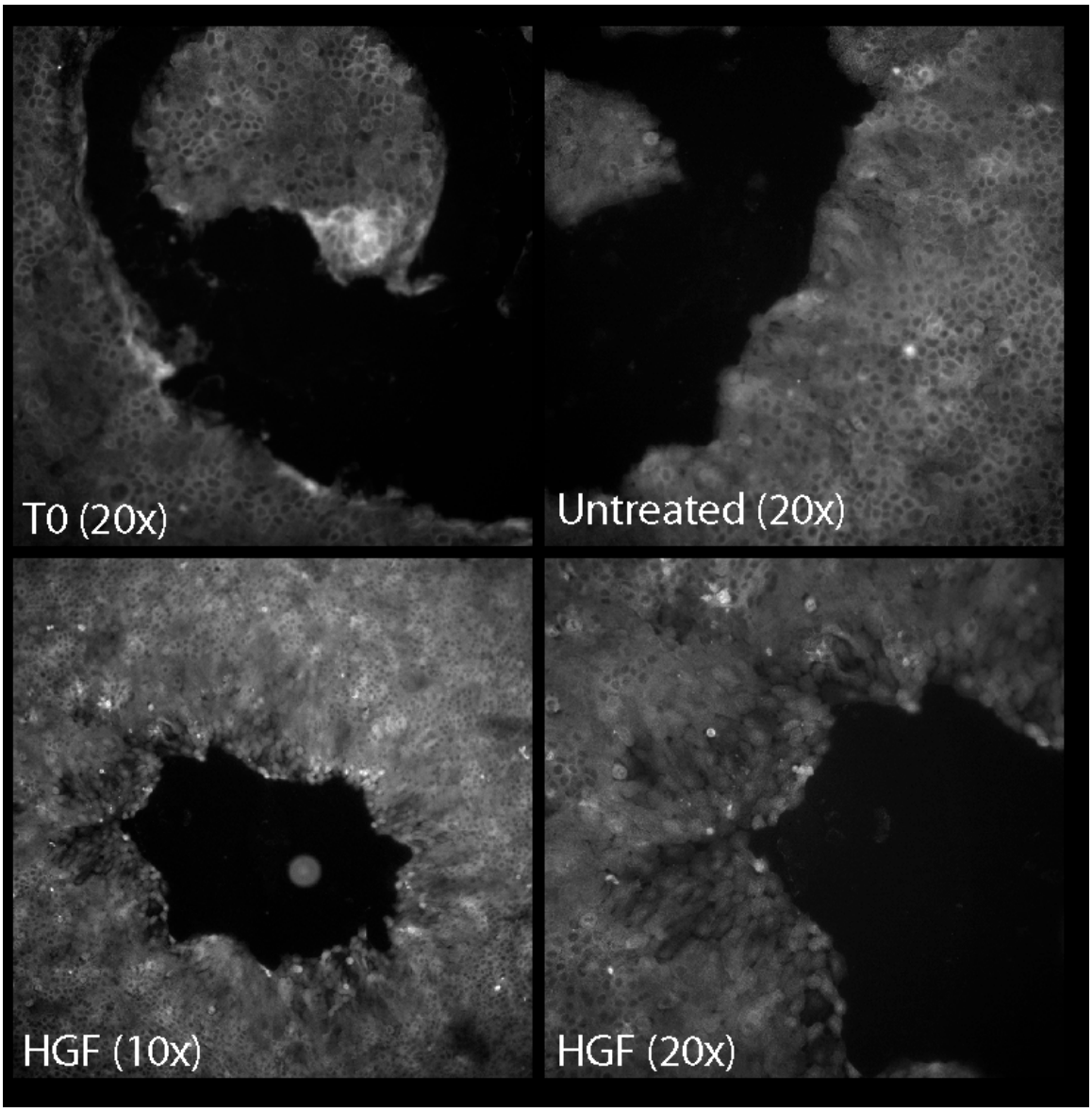

Figure 6. Nuclear Met is seen at the wound edge in A431 cells untreated and treated with HGF (50 ng/ml) after 24 hours. Top left: Untreated cells at time 0. Top right, Untreated cells 24 hours after wounding. Bottom left, HGF treatment 10x. Bottom right, HGF treatment 20x. Cells were stained with Met Ab CVD13 (Invitrogen) 1:500.

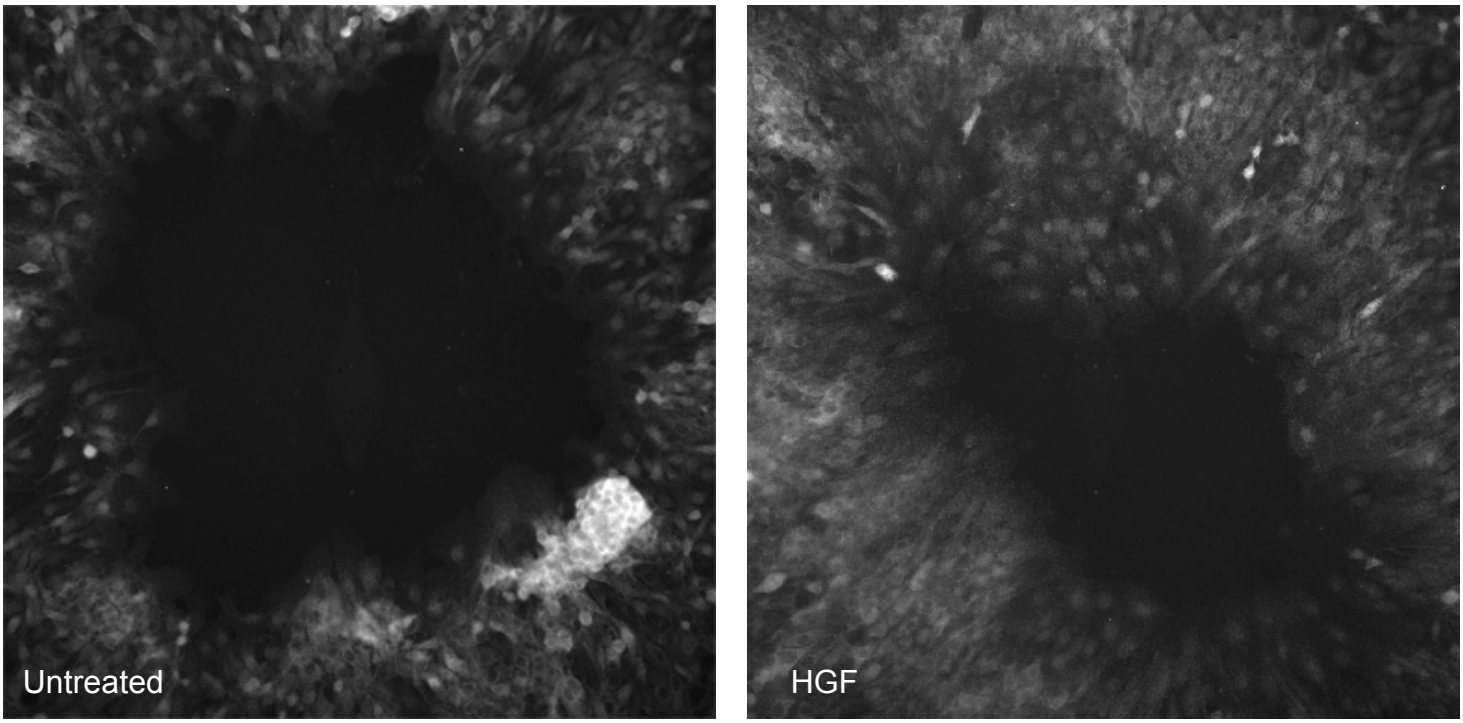

Figure 7. H1650 cells stained with phospho-Met (Cell Signaling Ab Y1234/1235) show nuclear Met is also phosphorylated after wounding for 8 hours either untreated (Left) or treated with HGF 50ng/ml (Right).

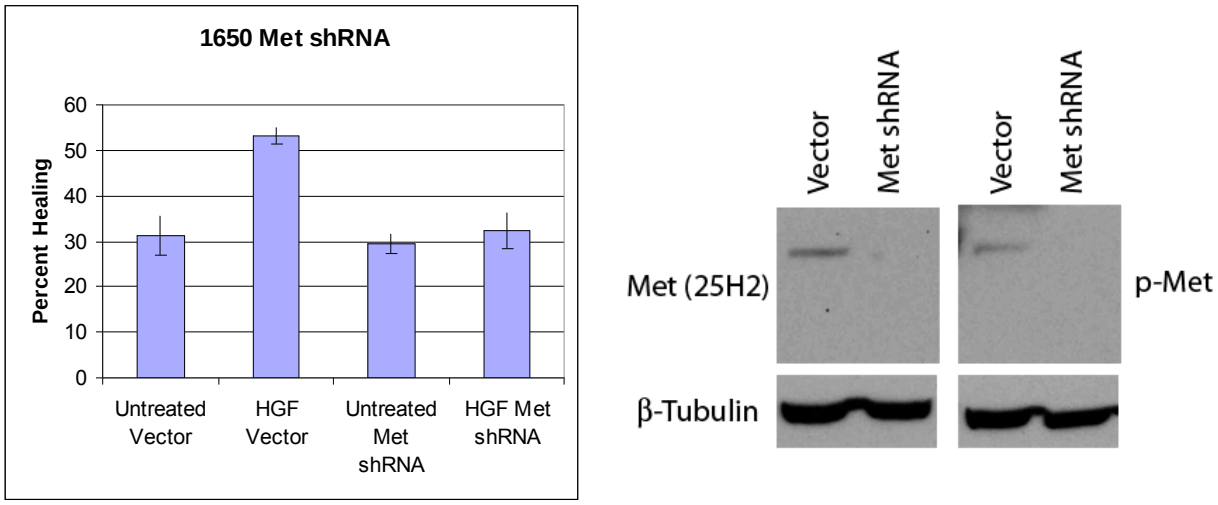

Figure 8. Left: Total wound healing response of H1650 control cells vs. Met shRNA knockdown cells untreated or stimulated with HGF. HGF stimulated healing response is abolished in knockdown cells. Right: WB of cell lysates shows efficacy of Met knockdown. Beta-tubulin is the loading control.

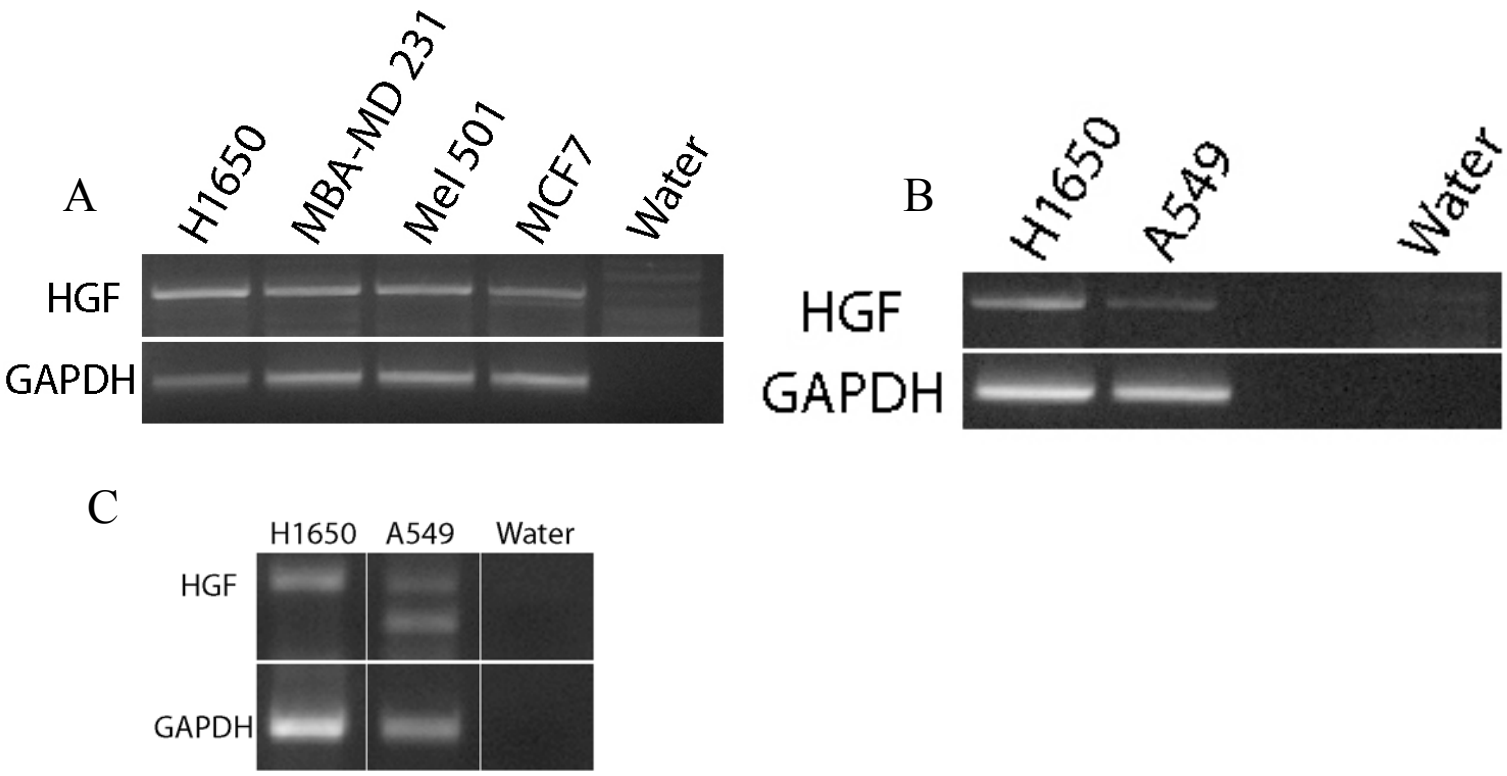

Figure 9. Total RNA was isolated from H1650, MBA-MD 231, Mel 501, MCF7, and A549 cell lines. cDNA was generated and used for PCR to determine HGF expression. The following HGF primers were used in A and B: Forward 5' ATCAGACACCACACCGGCACAAAT Reverse 5' GAAATAGGGCAATAATCCCAAGGAA with 2 min denature at 94°C, 1 min annealing at 53°C, and 1 min extension at 72°C cycled 40 times. GAPDH served as a positive control and water as a negative control. A second set of primers were used to confirm H1650 and A549 expression in C: Forward 5' TGTCGCCATCCCCTATGCAG and Reverse 5' GGAGTCACAAGTCTTCAACT.

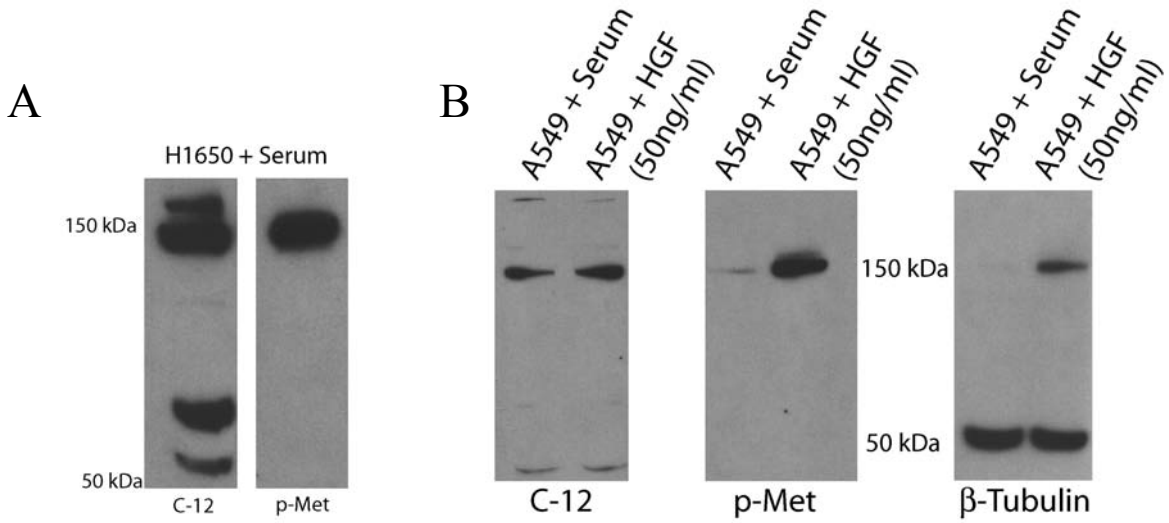

Figure 10. Met activation in H1650 (A) and A549 (B) cell lines. A: Total H1650 cell lysates grown in serum conditions were analyzed by Western blotting for total Met expression (C12 Invitrogen) and phospho-Met (Y1234/1235 Cell Signaling). Note activated Met in serum growth conditions without addition of exogenous HGF. B: Total cell lysates for A549 cells grown in serum or serum and stimulated with HGF (50ng/ml) for 30 min were analyzed by Western blotting for total Met and p-Met expression. Note activated Met after addition of exogenous HGF. Beta-tubulin served as a loading control.